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(54) Title: MITOCHONDRIAL DOSIMETER

(57) Abstract: Mitochondrial mutations occur as a product of contact of a person with an environmental pollutant. Mitochondrial mutations are readily detectable in body fluids. Measurement of mitochondrial mutations in body fluids can be used as a dosimeter to monitor exposure to the environmental pollutant. Mitochondrial mutations can also be detected in cancer patients. Probes and primers containing mutant mitochondrial sequences can be used to monitor patient condition.

MITOCHONDRIAL DOSIMETER

This application is a continuation-in-part of application Serial No. 09/377,856 filed August 20, 1999, which claims priority to provisional application Serial No. 60/097,307 filed August 20, 1998. The disclosure of these prior applications is expressly incorporated herein.

The U.S. Government retains certain rights in this invention due to funding as provided by grant CA43460 awarded by the National Institutes of Health.

TECHNICAL FIELD OF THE INVENTION

This invention is related to the field of environmental toxicology, in particular to methods for measuring the effects of environmental toxins.

BACKGROUND OF THE INVENTION

The human mitochondrial (mt) genome is small (16.5 kb) and encodes 13 respiratory chain subunits, 22 tRNAs and two rRNAs. Mitochondrial DNA is present at extremely high levels (10^3 - 10^4 copies per cell) and the vast majority of these copies are identical (homoplasmic) at birth (1). Expression of the entire complement of mt genes is required to maintain proper function of the organelle, suggesting that even slight alterations in DNA sequences could have profound effects (2). It is generally accepted that mtDNA mutations are generated endogenously during oxidative phosphorylation via pathways involving reactive oxygen species (ROS), but they can also be generated by external carcinogens or environmental toxins. These mutations may accumulate partially because mitochondria lack protective histones and highly efficient DNA repair mechanisms as seen in the nucleus (3). Recently several mtDNA mutations were found specifically in human colorectal cancer (4).

SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods of monitoring exposure of a person to an environmental pollutant.

It is another object of the present invention to provide a kit for monitoring exposure of a person to environmental pollutants.

It is an object of the invention to provide methods to aid in the detection of cancer or metastasis.

It is an object of the invention to provide probes and primers for detecting mitochondrial mutations.

It is an object of the invention to provide a method to aid in detecting the presence of tumor cells in a patient.

These and other objects of the invention are achieved by providing one or more of the embodiments described below. In one embodiment a method is provided for monitoring exposure of a person to an environmental pollutant. The presence of one or more mutations in mitochondrial DNA (mtDNA) in a body fluid of a person exposed to an environmental pollutant is determined at two or more time points. The amounts of mutations in mtDNA at different time points are compared. The amount of mutations correlates with amount of exposure to the environmental pollutant.

According to another embodiment another method is provided for monitoring exposure of a person to an environmental pollutant. The prevalence of one or more mutations in mitochondrial DNA (mtDNA) in a body fluid of a person exposed to an environmental pollutant is measured. A measured prevalence of one or more mutations in mtDNA of greater than 1% indicates clonal expansion of cells which harbor the one or more mutations in the person.

According to still another embodiment of the invention a method is provided for monitoring exposure of a person to an environmental pollutant. One or more mutations in a D-loop of mitochondrial DNA (mtDNA) in a body fluid of a person exposed to an environmental pollutant

are measured. The number of mutations in mtDNA correlates with exposure to the environmental pollutant.

According to yet another embodiment of the invention a kit is provided. The kit comprises one or more primers which hybridize to a mitochondrial D-loop for making a primer extension product. In addition, the kit contains written material identifying mutations which are found in the D-loop as a result of exposure to one or more environmental pollutants.

According to another embodiment of the invention an oligonucleotide probe is provided. The probe comprises a sequence of at least 10 contiguous nucleotides of a human mitochondrial genome. The probe can optionally contain at least 12, 14, 16, 18, 20, 22, 24, 26, or 30 such contiguous nucleotides. The oligonucleotide comprises a mutation selected from the group consisting of: a mutation selected from the group consisting of: T̄C at nucleotide 114; ΔC at nucleotide 302; C̄A at nucleotide 386; insert T at nucleotide 16189; ĀC at nucleotide 16265; ĀT at nucleotide 16532; C̄T at nucleotide 150; T̄C at nucleotide 195; ΔC at nucleotide 302; C̄A at nucleotide 16183; C̄T at nucleotide 16187; T̄C at nucleotide 16519; ḠA at nucleotide 16380; ḠA at nucleotide 75; insert C at nucleotide 302; insert CG at nucleotide 514; T̄C at nucleotide 16172; C̄T at nucleotide 16292; ĀG at nucleotide 16300; A G at nucleotide 10792; C T at nucleotide 10793; C T at nucleotide 10822; A G at nucleotide 10978; A G at nucleotide 11065; G A at nucleotide 11518; C T at nucleotide 12049; T C at nucleotide 10966; G A at nucleotide 11150; G A at nucleotide 2056; T C at nucleotide 2445; T C at nucleotide 2664; T C at nucleotide 10071; T C at nucleotide 10321; T C at nucleotide 12519; Δ 7 amino acids at nucleotide 15642; G A at nucleotide 5521; G A at nucleotide 12345; T̄Y C substitution at position 710; T̄Y C substitution at position 1738; T̄Y C substitution at position 3308; ḠY A substitution at position 8009; ḠY A substitution at position 14985; T̄Y C substitution at position 15572; ḠY A substitution at position 9949; T̄Y C substitution at position 10563; ḠY A substitution at

position 6264; A insertion at position 12418; TÝC substitution at position 1967; TÝA substitution at position 2299; and G A at nucleotide 3054.

According to another aspect of the invention an oligonucleotide primer is provided. It comprises a sequence of at least 10 contiguous nucleotides of a human mitochondrial genome. The primer can optionally contain at least 12, 14, 16, 18, 20, 22, 24, 26, or 30 such contiguous nucleotides. The oligonucleotide comprises a mutation selected from the group consisting of: a mutation selected from the group consisting of: TÖC at nucleotide 114; ΔC at nucleotide 302; CÖA at nucleotide 386; insert T at nucleotide 16189; AÖC at nucleotide 16265; AÖT at nucleotide 16532; CÖT at nucleotide 150; TÖC at nucleotide 195; ΔC at nucleotide 302; CÖA at nucleotide 16183; CÖT at nucleotide 16187; TÖC at nucleotide 16519; GÖA at nucleotide 16380; GÖA at nucleotide 75; insert C at nucleotide 302; insert CG at nucleotide 514; TÖC at nucleotide 16172; CÖT at nucleotide 16292; AÖG at nucleotide 16300; A G at nucleotide 10792; C T at nucleotide 10793; C T at nucleotide 10822; A G at nucleotide 10978; A G at nucleotide 11065; G A at nucleotide 11518; C T at nucleotide 12049; T C at nucleotide 10966; G A at nucleotide 11150; G A at nucleotide 2056; T C at nucleotide 2445; T C at nucleotide 2664; T C at nucleotide 10071; T C at nucleotide 10321; T C at nucleotide 12519; Δ 7 amino acids at nucleotide 15642; G A at nucleotide 5521; G A at nucleotide 12345; TÝC substitution at position 710; TÝC substitution at position 1738; TÝC substitution at position 3308; GÝA substitution at position 8009; GÝA substitution at position 14985; TÝC substitution at position 15572; GÝA substitution at position 9949; TÝC substitution at position 10563; GÝA substitution at position 6264; A insertion at position 12418; TÝC substitution at position 1967; TÝA substitution at position 2299; and G A at nucleotide 3054.

Another aspect of the invention is a method to aid in detecting the presence of tumor cells in a patient. The presence of a single basepair mutation is detected in a mitochondrial genome of a cell sample of a

patient. The mutation is found in a tumor of the patient but not in normal tissue of the patient. The tumor is not a colorectal tumor. The patient is identified as having a tumor if one or more single basepair mutations are determined in the mitochondrial genome of the cell sample of the patient.

Yet another embodiment of the invention is provided by another method to aid in detecting the presence of tumor cells in a patient. The presence of a mutation is determined in a D-loop of a mitochondrial genome of a cell sample of a patient. The mutation is found in a tumor of the patient but not in normal tissue of the patient. The patient is identified as having a tumor if one or more single basepair mutations are determined in the mitochondrial genome of the cell sample of the patient.

According to still another aspect of the invention a method is provided to aid in detecting the presence of tumor cells in a patient. The presence of a single basepair mutation is determined in a mitochondrial genome of a cell sample of a patient. The mutation is found in a cancer of the patient but not in normal tissue of the patient. The cancer is selected from the group of cancers consisting of: lung, head and neck, bladder, brain, breast, lymphoma, leukaemia, skin, prostate, stomach, pancreas, liver, ovarian, uterine, testicular, and bone. The patient is identified as having a tumor if one or more single basepair mutations are determined in the mitochondrial genome of the cell sample of the patient.

According to still another aspect of the invention a method is provided to aid in detecting the presence of tumor cells in a patient. The presence of a single basepair mutation is determined in a mitochondrial genome of a cell sample of a patient. The mutation is found in a tumor of the patient but not in normal tissue of the patient. The cancer is selected from the group of cancers consisting of: lung, head and neck, and bladder. The patient is identified as having a tumor if one or more single basepair mutations are determined in the mitochondrial genome of the cell sample of the patient.

Another embodiment of the invention provides a method to aid in detecting the presence of tumor cells in a patient. The presence of a

mutation in a mitochondrial genome of a cell sample of a patient is determined. The mutation is selected from the group consisting of: **T** at nucleotide 114; **ΔC** at nucleotide 302; **C** at nucleotide 386; insert **T** at nucleotide 16189; **A** at nucleotide 16265; **A** at nucleotide 16532; **C** at nucleotide 150; **T** at nucleotide 195; **ΔC** at nucleotide 302; **C** at nucleotide 16183; **C** at nucleotide 16187; **T** at nucleotide 16519; **G** at nucleotide 16380; **G** at nucleotide 75; insert **C** at nucleotide 302; insert **CG** at nucleotide 514; **T** at nucleotide 16172; **C** at nucleotide 16292; **A** at nucleotide 16300; **A** **G** at nucleotide 10792; **C** **T** at nucleotide 10793; **C** **T** at nucleotide 10822; **A** **G** at nucleotide 10978; **A** **G** at nucleotide 11065; **G** **A** at nucleotide 11518; **C** **T** at nucleotide 12049; **T** **C** at nucleotide 10966; **G** **A** at nucleotide 11150; **G** **A** at nucleotide 2056; **T** **C** at nucleotide 2445; **T** **C** at nucleotide 2664; **T** **C** at nucleotide 10071; **T** **C** at nucleotide 10321; **T** **C** at nucleotide 12519; **Δ** 7 amino acids at nucleotide 15642; **G** **A** at nucleotide 5521; **G** **A** at nucleotide 12345; **T** **C** substitution at position 710; **T** **C** substitution at position 1738; **T** **C** substitution at position 3308; **G** **A** substitution at position 8009; **G** **A** substitution at position 14985; **T** **C** substitution at position 15572; **G** **A** substitution at position 9949; **T** **C** substitution at position 10563; **G** **A** substitution at position 6264; **A** insertion at position 12418; **T** **C** substitution at position 1967; **T** **A** substitution at position 2299; and **G** **A** at nucleotide 3054. The patient is identified as having a tumor if one or more mutations are determined in the mitochondrial genome of the cell sample of the patient.

These and other embodiments provide the art with non-invasive tools for monitoring exposure to and the effects of environmental pollutants on the human body as well as early detection methods for cancer and metastasis.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Schematic representation of a linearized mt genome. Hatched bars indicate the regions sequenced in this study and solid bars indicate the positions of tRNAs (transfer RNAs). rRNA = ribosomal RNA, *ND* = NADH dehydrogenase, *COX* = cytochrome c oxidase, *Cyt b* = cytochrome b, *ATPase* = ATP synthase.

Fig. 2. Sequence detection of mutated mtDNAs in samples from tumors and bodily fluids. (Fig. 2A) The mt mutation was analyzed by direct sequencing of the tumor (T), normal (N), and corresponding urine (U) DNAs of bladder cancer patient #799. The arrow indicates a single nucleotide change (G(A) at 2056 np in the 16S rRNA gene. (Fig. 2B and Fig. 2C) Examples of somatic mutations in head and neck cancers. Both mutations at 16172 np (B) and 10822 np (C) were detected from saliva (S) samples from patients #1680 and #1708, respectively. (Fig. 2D) Mutated mtDNA at 2664 np was not detected by sequence analysis in the paired BAL fluid (B), obtained from lung cancer patient #898.

Fig. 3. Oligonucleotide-mismatch ligation assay (22) to detect mtDNA mutations in BAL. The arrows identify mutated mt sequences at 12345 np within tRNA (Fig. 3A) and at 2664 np (Fig. 3B) within 16S rRNA in the tumor DNA. More dilute signals are seen in the corresponding BAL (B) samples with no detectable signal from the paired normal (N) tissue.

Fig. 4. Highly enriched mutated mtDNA in BAL samples from lung cancer patients. Oligonucleotide (oligo) specific hybridization detected ~ 2000 plaques containing WT p53 clones in the BAL from patient #1113, and only two plaques (2/2000=0.1%) with the p53 gene mutation (Fig. 4A) were found in the primary tumor. The same BAL sample demonstrated a much greater enrichment of mutated mtDNA; 445 plaques contained mtDNA mutations (Fig. 4A) at 16159 np (445/2000=22.3%; 220-fold) compared to approximately 1500 WT clones. A similar enrichment was seen in patient #1140 where oligo specific

hybridization detected 12 p53 mutant plaques among 437 WT clones (2.7 %, Fig. 4B), while mutant mtDNA at 16380 np (Fig. 4B) represented over 50% of the plaques (52.3%, 460/880; 19-fold) amplified from mtDNA.

Fig. 5. Pseudoclonal selection of mtDNA. A mitochondrial genome gains some replicative advantage due to a somatic mutation (such as in the D-loop region), leading to a dominant mitochondrial genotype (step 1). This mitochondrion can gain additional replicative advantage through nuclear influences: for example, a mutated sequence gains a higher binding affinity to nuclear-encoded mitochondrial trans-acting factors (step 2). Due to its stochastic segregation together with the clonal expansion of a neoplastic cell driven by nuclear mutations, mutated mitochondria overtake the entire population of tumor cells (step 3).

BRIEF DESCRIPTION OF THE TABLES

Table 1 provides a summary of mutations in mitochondria of colorectal tumors.

Table 2 provides a summary of mutations in mitochondria of bladder, lung, head and neck tumors.

Table 3 provides a summary of new polymorphisms in mitochondria of bladder, lung, head and neck tumors.

DETAILED DESCRIPTION

It is a discovery of the present inventors that mitochondrial DNA mutations can be monitored non-invasively and sensitively and used as an indicator of environmental pollutants. It is shown below that these mutations are more prevalent in body samples than nuclear mutations, and thus are detected more sensitively. Mitochondrial mutations can be monitored over time to detect changes in the amount of exposure to pollutants. In addition, the prevalence of the mitochondrial mutation in the sample indicates whether clonal proliferation has occurred. Finally, the D-loop has been identified as a hotspot of mutations within the mitochondrial genome.

Mitochondrial mutations are determined with reference to wild-type human mitochondrial sequence. Sequence information can be found at the

website <http://www.gen.emory.edu/mitomap.html> and at SEQ ID NO: 1. However, some differences between a sample sequence and a documented wild-type sequence can be polymorphisms, not mutations. Table 3 provides a number of new polymorphisms. Other polymorphisms can be found in references 2 and 8. Polymorphisms can be distinguished from somatic mutations by comparing the sequence in the sample to the corresponding sequence in a normal body tissue of the same person. If the same variant sequence is found in the sample as in the normal body tissue it is a polymorphism. Normal tissues can be paraffin-embedded. It has been found by the present inventors that mitochondrial DNA which is paraffin-embedded remains more highly intact and amplifiable than genomic DNA. Amplifiable regions of mitochondrial DNA may be from 10 bp to about 4 kb, desirably 2 kb to 4 kb or 10 bp to about 2 kb. Other suitable sources of reference mtDNA are blood, serum, or plasma of the human being tested.

Suitable bodily fluids for testing according to the present invention include saliva, sputum, urine, and bronchoalveolar lavage (BAL). These can be collected as is known in the art. People who are prime candidates for testing and supplying such bodily fluids are those who have been episodically, periodically or chronically exposed to environmental pollutants. These include without limitation cigarette smoke, biological toxins, such as aflatoxin, cholera toxin, and botulinum toxin, radiation including UV irradiation, industrial wastes, chemicals, water-borne or air-borne pollutants, and drugs. The environmental pollutant can be known, suspected, or unidentified, as the assay depends on the effect and not on the identity of the pollutant.

The inventors have found that there are certain characteristics of the mutations which are found in mitochondrial DNA. Many mutations are found in sequences which do not encode proteins. These include the D-loop region (*i.e.*, nucleotides 16024-526), the 16S RNA gene, and the tRNA genes. Furthermore, even where the mutations do occur in protein coding regions, they often result in silent mutations which do not affect the encoded amino acids. Other regions frequently affected include the genes

for NADH dehydrogenase 4, NADH dehydrogenase 3, NADH dehydrogenase 5, and cytochrome B.

Mutation detection can be done according to any methodology which is known in the art for determining mutations. These include without limitation, nucleotide sequencing, hybridization, amplification, PCR, oligonucleotide mismatch ligation assays, primer extension assays, heteroduplex analysis, allele-specific amplification, allele-specific primer extension, SCCP, DGGE, mass spectroscopy, high pressure liquid chromatography, and combinations of these techniques.

Prevalence of a particular mutation according to the present invention can be used to monitor clonal expansion. Mutations which are present in greater than 1% of the mitochondrial DNA present in a sample have may have conferred a growth advantage on the cells harboring them. Even if no growth advantage is conferred by the mutation itself, the mutation serves as a marker for a clone which is expanding relative to the population of cells in the sample. Clonal expansion can be measured over time to monitor the growth of the clone or to monitor the efficacy of anti-proliferative agents which can be considered environmental pollutants, according to the present invention.

The inventors have also found that the presence of subtle mutations in the mitochondrial genome can be used as a means to trace the presence, spread, metastasis, growth, or recurrence of a tumor in a patient. Such subtle mutations include single basepair substitutions, single basepair insertions, and single basepair deletions. Single basepair substitutions can be either transitions or transversions, although the former are more frequent. Detection of such mutations can be useful to screen for the initial appearance of a tumor as well as the recurrence of a previously identified tumor. The methods are particularly suited to monitor anti-cancer therapy, recurrence, metastasis, and completeness of surgical removals.

A single basepair substitution is the substitution of a single nucleotide base with a different nucleotide base at the same position, with the corresponding substitution of the complementary base on the other

strand of the DNA. While any single basepair substitution is conceivable within the scope of the invention, the most frequently encountered substitutions are those which are consistent with endogenous oxidative damage, such as T to C or G to A transitions, or which are consistent with a variety of external carcinogens which cause a variety of types of mutations. The mutations can appear in protein coding or non-coding regions or in regions which encode ribosomal or transfer RNAs.

The homoplasmic or near homoplasmic property of most mutant mitochondrial genomes from tumors permits the ready detection of such mutations within a sample of mitochondrial DNA from a patient. Homoplasmic mutations are those which appear in essentially all of the copies of the mitochondrial genome within a given cell or tissue. However, heteroplasmic mutations, which are those appearing in only a fraction of the mitochondrial genomes of a cell or tissue, are also suitable for use with the invention.

Any cell sample can be tested from a patient who has cancer or is suspected of having cancer. Suitable cell samples include, but are not limited to, tissue from a growth suspected or known to be cancerous, tissue adjacent to a resection of a tumor, and tissue distant from the site of a tumor, such as lymph nodes which are suspected of bearing metastatic cells. Cells can also be obtained from bodily fluids or secretions, e.g., blood, urine, sputum, saliva, or feces, which may contain cancerous cells or metastatic cells. Cell samples can also be collected from other bodily secretions and tissues as is known in the art. A cell sample can be collected from suspected or known cancerous tissue or from bodily fluids or secretions harboring cancer cells as well as from suspected or known normal tissue or bodily fluids or secretions harboring normal cells.

In order to detect mutations of the mitochondrial genome from a cell sample of a patient, mitochondrial DNA can be isolated from the cell sample using any method known in the art. One way of identifying subtle mutations involves sequencing the mitochondrial DNA. This can be done according to any method known in the art. For example, isolated

mitochondrial DNA can be cleaved using endonucleases into overlapping fragments of appropriate size for sequencing, *e.g.*, about 1-3 kilobases in length, followed by polymerase chain reaction (PCR) amplification and sequencing of the fragments. Examples of DNA sequencing methods are found in Brumley, R.L. Jr., and Smith, L.M., 1991, Rapid DNA sequencing by horizontal ultrathin gel electrophoresis, *Nucleic Acids Res.* 19:4121-4126 and Luckey, J.A., Drossman, H., Kostihka, T.; and Smith, L.M., 1993, High-speed DNA sequencing by capillary gel electrophoresis, *Methods Enzymol.* 218:154-172. Amplification methods such as PCR can be applied to samples as small as a single cell and still yield sufficient DNA for complete sequence analysis. The combined use of PCR and sequencing of mitochondrial DNA is described in Hopgood, R., Sullivan, K.M., and Gill, P., 1992, Strategies for automated sequencing of human mitochondrial DNA directly from PCR products, *Biotechniques* 13:82-92 and Tanaka, M., Hayakawa, M., and Ozawa, T., 1996, Automated sequencing of mitochondrial DNA, *Methods Enzymol.* 264:407-21.

Mutations can first be identified by comparison to sequences present in public databases for human mitochondrial DNA, *e.g.*, at <http://www.gen.emory.edu/mitomap.html> and at SEQ ID NO: 1. Any single basepair substitution identified in the sample DNA compared to a normal sequence from a database can be confirmed as being a somatic mutation as opposed to a polymorphic variant by comparing the sample mitochondrial DNA or sequences obtained from it to control cell mitochondrial DNA from the same individual or sequences obtained from it. Control cells are isolated from other apparently normal tissues, *i.e.*, tissues which are phenotypically normal and devoid of any visible, histological, or immunological characteristics of cancer tissue. A difference between the sample and the control identifies a somatic mutation which is associated with the tumor.

An alternative to serially sequencing the entire mitochondrial genome in order to identify a single basepair substitution is to use hybridization of the mitochondrial DNA to an array of oligonucleotides.

Hybridization techniques are available in the art which can rapidly identify mutations by comparing the hybridization of the sample to matched and mismatched sequences which are based on the human mitochondrial genome. Such an array can be as simple as two oligonucleotide probes, one of whose sequence matches the wild-type or mutant region containing the single base substitution (matched probe) and another whose sequence includes a single mismatched base (mismatch control probe). If the sample DNA hybridizes to the matched probe but not the mismatched probe, it is identified as having the same sequence as the matched probe. Larger arrays containing thousands of such matched/mismatched pairs of probes on a glass slide or microchip ("microarrays" or "gene chips") are available which are capable of sequencing the entire mitochondrial genome very quickly. Such arrays are commercially available. Review articles describing the use of microarrays in genome and DNA sequence analysis and links to their commercial suppliers are available at www.gene-chips.com.

The invention can be used to screen patients suspected of having cancer for the presence of tumor cells. A cell sample is first obtained from a suspected tumor of the patient, or is obtained from another source such as blood or lymph tissue, for example, if metastasis is suspected. The cell sample is tested to determine the presence of a single basepair mutation in mitochondrial DNA from the cell sample using the techniques outlined above. Optionally, a cell sample from normal, non-cancerous cells or tissue of the patient is also obtained and is tested for the presence or absence of a single basepair mutation in mitochondrial DNA. If a single basepair mutation is determined which is not present in a cell sample from normal tissue of the patient, then the mutation is a somatic mutation and the presence of tumor cells in the patient is indicated. If one or more single basepair mutations are determined in the mitochondrial genome of the cell sample of the patient, then the patient is identified as having a tumor. As in any diagnostic technique for cancer, to confirm or extend the diagnosis, further diagnostic techniques may be warranted. For example,

conventional histological examination of a biopsy specimen can be performed to detect the presence of tumor cells, or analysis of a tumor-specific antigen in a blood or tissue sample can be performed.

The method outlined above can be practiced either in the situation where the somatic mutation is previously known or previously unknown. The method can be practiced even in the absence of prior knowledge about any particular somatic mutation. The method can also be carried out subsequent to the discovery of a somatic mutation in a mitochondrial genome of a cell of the patient or of another patient. In this case, a previous association of the somatic mutation with the presence of a tumor in the patient or in another patient strongly indicates the presence of tumor cells in the patient. It may also indicate the recurrence of a tumor or the incomplete prior removal of cancerous tissue from the patient.

The effectiveness of therapy can be evaluated when a tumor has already been identified and found to contain a single basepair substitution in the mitochondrial genome. Once a single basepair mutation has been identified in the mitochondrial DNA of a tumor of the patient, further tumor cells can be detected in tissue surrounding a resection or at other sites, if metastasis has occurred. Using the methods outlined above, the recurrence of the tumor or its incomplete removal can be assessed. Similarly, if a tumor has been treated using a non-surgical method such as chemotherapy or radiation, then the success of the therapy can be evaluated at later times by repeating the analysis. The step for determining the presence of a single basepair mutation in a mitochondrial genome of a cell sample of a patient can be performed 1, 2, 3, 4, 5, 6, 8, 10, or more times in order to monitor the development or regression of a tumor or to monitor the progress or lack of progress of therapy undertaken to eliminate the tumor.

Upon repeated analyses, the step for determining the presence of a single basepair mutation is simplified, because only a well defined and limited region of the genome need be sequenced. Using the hybridization method, for example, it is possible to evaluate the presence of the mutation with only a single matched/mismatched pair of oligonucleotide probes in

the array. In the event that a mixture of genotypes is observed, it is possible to obtain quantitative information about the relative amount of each mitochondrial genotype using techniques known to the art, e.g., hybridization. Quantitative analysis can reveal changes in the relative proportion of tumor to normal cells in a tissue over time or in response to therapy.

The following examples are provided to demonstrate certain aspects of the invention but they do not define the scope of the invention.

EXAMPLE 1

This example demonstrates detection of mt mutations in tissue samples.

To determine whether mt mutations could be identified in cancer other than colorectal cancer, we studied primary bladder (n = 14), head and neck (n = 13), and lung (n = 14) tumors (5). Eighty percent of the mt genome of all the primary tumor samples was PCR-amplified (6) and sequenced manually (Fig.1). Tumor mtDNA was compared to mtDNA from paired blood samples in all cases, and mtDNA from corresponding normal tissue when available (7). Of the 292 sequence variants detected, 196 were previously recorded polymorphisms (2, 8), while 57 were novel polymorphisms (Table 3). The remaining 39 variants were acquired (somatic) mutations identified in 64% (9/14) of the bladder cancer, 46% (6/13) of the head and neck cancer, and 43% (6/14) of the lung cancer patients (Table 2). Most of these mutations were T-to-C and G-to-A base transitions, indicating possible exposure to ROS-derived mutagens (9). Similar to the previous observation by Polyak *et al.* (Table 1; 4), the majority of the somatic mutations identified here were also homoplasmic in nature. In addition, several of the bladder and head and neck cancers studied here (Table 2) had multiple mutations implying possible accumulation of mtDNA damage.

In the bladder tumors, mutation hot spots were primarily in the NADH dehydrogenase subunit 4 (ND4) gene (35%), and in the displacement-loop (D-loop) region (30%). The D-loop region is a critical site for both replication and expression of the mt genome since it contains

the leading-strand origin of replication and the major promoters for transcription (10). Many (73%) of the mutations identified within protein-coding regions were silent, except for a (Val \rightarrow Ile) substitution in the NADH-dehydrogenase subunit 3 (*ND3*) and a 7-amino-acid deletion in cytochrome b (*Cyt b*). The D-loop region was also commonly mutated in head and neck cancer (67%). Two of the head and neck tumors (22%) contained mutations in the *ND4* gene at nucleotide pairs (nps) 10822 and 11150, resulting in amino acid substitutions of Thr \rightarrow Met and Ala \rightarrow Thr, respectively. A similar tendency was observed in lung cancers, demonstrating a high concentration of mutations in the D-loop region (70%).

EXAMPLE 2

This example demonstrates detection of mt mutations in bodily fluids.

We hypothesized that the homoplasmic nature of these mutations would make them readily detectable in paired bodily fluids. To test this, we extracted and directly amplified mtDNA from urine samples from patients diagnosed with bladder cancer. All three corresponding urine samples available in this study contained the mutant mtDNA derived from tumor tissues. For example, the mtDNA from a urine sample of bladder cancer patient #799 showed the same nucleotide transition (G \rightarrow A) as seen in the tumor (Fig 2A). In all cases, the urine sample contained a relatively pure population of tumor-derived mtDNA, comparable to that of the micro-dissected tumor sample. Consistent with this observation, saliva samples obtained from head and neck cancer patients contained no detectable wild-type (WT) signals (Fig. 2B, and 2C). By sequence analysis alone, we were able to detect mtDNA mutations in 67% (6/9) of saliva samples from head and neck cancer patients. In lung cancer cases, we were initially unable to identify mutant bands from paired bronchoalveolar lavage (BAL) fluids because of the significant dilution of neoplastic cells in BAL fluid (11), (Fig 2D). We, thus, applied a more sensitive oligonucleotide-mismatch ligation assay to detect mutated mtDNA. As shown in Fig. 3A and 3B, both lung cancer mutations

(arrows) were confirmed in tumor mtDNA with more dilute signals in the corresponding BAL samples, and no signal in the corresponding normal tissues. Again, we detected the majority of mtDNA mutations (8/10) in BAL fluids with the exception of two cases where the ligation assays were not feasible due to the sequence compositions (16183 and 302 nps) adjacent to the mutations.

EXAMPLE 3

This example demonstrates the enrichment of mitochondrial mutant DNA in samples relative to nuclear mutant DNA.

To quantitate this neoplastic DNA enrichment, we compared the abundance of mt gene mutations to that of nuclear-encoded p53 mutations in bodily fluids using a quantitative plaque assay. Nuclear and mt fragments that contained a mutated sequence were PCR-amplified and cloned for plaque hybridization (12). Two BAL samples from lung cancer patients were chosen for analysis because they had mutations in both the mt and nuclear genomes. For p53 mutations, the percentages of neoplastic cells among normal cells for patients #1113 and #1140 were 0.1 and 3.0 %, respectively. Remarkably, the abundance of the corresponding mutated mtDNA (MT) was 22% and 52% when compared to the wild-type (WT) mt sequence (Fig. 4). This enrichment of mtDNA is presumably due to the homoplasmic nature of these mutations and the high copy number of mt genomes in cancer cells. Enrichment was further suggested by our observations in head and neck paraffin samples where we were able to PCR-amplify 2-3 kb fragments of mtDNA, whereas we were unable to amplify nuclear p53 gene fragments of over 300 bp.

A role for mitochondria in tumorigenesis was implicated when tumor cells were found to have an impaired respiratory system and high glycolytic activity (13,14). Recent findings elucidating the role of mitochondria in apoptosis (15) and the high incidence of mtDNA mutations in colon cancer (4) further support the original hypothesis of mitochondrial participation in the initiation and progression of cancer. Although further investigation is needed to define the functional significance of mt

mutations, our data clearly show that those mutations are frequent and present at high levels in all of the tumor types examined.

The homoplasmic nature of the mutated mitochondria remains puzzling. It is estimated that each cell contains several hundred-to-thousands of mitochondria and that each mitochondrion contains 1-10 genomes (16). Conceivably, certain mutated mtDNAs may gain a significant replicative advantage. For example, mutations in the D-loop regulatory region might alter the rate of DNA replication by modifying the binding affinity of important trans-acting factors. Mitochondria that undergo the most rapid replication are likely to acquire more DNA damage, leading to an accumulation of mutational events. Although the mechanism may vary for other mutations (such as silent mutations in the *ND4* gene), the accumulation of a particular mtDNA mutation may become more apparent during neoplastic transformation. Even subtle mtDNA mutations may also gain significant replicative advantage, perhaps through interactions with important nuclear factors. Homoplasmic transformation of mtDNA was observed in small populations of cells in other non-neoplastic, but diseased tissues (17), sometimes associated with aging (18). We hypothesize that, in contrast to classic clonal expansion, the process may occur as "pseudoclonal" selection where stochastic segregation of mitochondria (16) together with neoplastic clonal expansion driven by nuclear mutations lead to a homogeneous population of a previously "altered" mitochondrion (Fig. 5).

The large number of mt polymorphisms identified here and elsewhere (2) likely reflects the high mutation rate of mtDNA, which is thought to be caused mainly by high levels of ROS (19). In agreement with this, our data imply that constitutive hypervariable areas such as the D-loop region represent somatic mutational hot spots. As further mutations are tabulated in primary tumors, DNA-chip technology can be harnessed to develop high-throughput analyses with sufficient sensitivity (20, 21). Due to its high copy number, mtDNA may provide a distinct advantage over

other nuclear genome based methods for cancer and environmental pollutant detection.

Literature Cited

1. R.N. Lightowers, P.F. Chinnery, D.M. Turnbull, N. Howell, Trends Genet 13, 450 (1997).
2. MITOMAP: A Human Mitochondrial Genome Database. Center for Molecular Medicine, Emory University, Atlanta, GA, USA.
<http://www.gen.emory.edu/mitomap.html>
3. D.L. Croteau and V.A. Bohr; J. Biol. Chem. 272, 25409 (1997).
4. K. Polyak *et al.*, Nature Genet. 20, 291 (1998).
5. Paired normal and tumor specimens along with blood and bodily fluids were collected following surgical resections with prior consent from patients in The Johns Hopkins University Hospital. Tumor specimens were frozen and micro-dissected on a cryostat so that the tumor samples contained greater than 70% neoplastic cells. DNA from tumor sections was digested with 1% SDS/Proteinase K, extracted by phenol-chloroform, and ethanol precipitated. Control DNA from peripheral lymphocytes, matched normal tissues, from urine, saliva, and BAL fluid were processed in the same manner as described in (11).
6. Mitochondrial DNAs were amplified using overlapping primers (4) in PCR buffer containing 6% DMSO. Approximately 5-20 ng of genomic DNA was subjected to the step-down PCR protocol: 94°C 30 sec, 64°C 1 min, 70°C 3 min, 3 cycles, 94°C 30 sec, 61°C 1min, 70°C 3 min, 3 cycles, 94°C 30 sec, 58°C 1 min, 70°C 3.5 min 15 cycles, 94°C 30 sec, 57°C 1 min, 70°C 3.5 min, 15 cycles, and a final extension at 70°C for 5 min. PCR products were gel-purified using a Qiagen gel extraction

kit (Qiagen) and sequence reactions were performed with Thermosequenase (Perkin-Elmer) using the cycle conditions (95°C 30 sec, 52°C 1 min, and 70°C 1 min for 25 cycles).

7. Corresponding normal tissues from 4 patients (#874, #915, #1684, and #1678) were available and DNA was extracted from paraffin samples as described previously (9).
8. R.M. Andrew *et al.*, *Nature Genet.* 23 147, (1999)
9. J. Cadet, M. Berger, T. Douki, J.L. Ravanat, *Rev. Physiol. Biochem. Pharmacol.* 131, 1 (1997).
8. J.W. Taanman, *Biochimica et Biophysica Acta.* 1410, 103 (1999).
9. S.A. Ahrendt *et al.*, *J. Natl. Cancer Inst.* 91, 332 (1999).
10. Subcloning of PCR fragments into phage vector was performed according to the manufacturer's instructions (Stratagene). Titered plaques were plated and subjected to hybridization using tetramethylammonium chloride (TMAC) as a solvent. Positive signals were confirmed by secondary screenings. Oligonucleotides (Oligos) used for this assay were as follows; for patient #1113, p53 and mtDNA sequence alterations were detected using oligos containing either WT- (p53: 5'-GTATTTGGATGTCAGAAACACTT-3' (SEQ ID NO: 2)/ mtDNA: 5'-ACTTCAGGGTCATAAAGCC-3' (SEQ ID NO: 3)) or MT- (p53: 5'-GTATTTGGATGTCAGAAACACTT-3' (SEQ ID NO: 4)/ mtDNA: 5'-ACTTCAGGCCATAAAGCC-3' (SEQ ID NO: 5)) sequences, respectively. For patient #1140, oligos 5'-ACCCCGCGTCCCGGCCATGGCC-3' (SEQ ID NO: 6) and

5'-ACCCGCGTCCTGCCATGGCC-3' (SEQ ID NO: 7) were used to detect WT and MT sequences, respectively.

11. O. Warburg, *Science* 123, 309 (1956).
12. J.W. Shay and H. Werbin, *Mut. Res.* 186, 149 (1987).
13. D.R. Green and J.C. Reed, *Science* 281, 1309 (1999).
14. D.C. Wallace, *Annu. Rev. Biochem.* 61, 1175 (1992).
15. D.C. Wallace, *Proc. Natl. Acad. Sci. USA* 91, 8746 (1994).
16. K. Khrapko *et al.*, *N.A.R.* 27, 2434 (1999).
17. C. Richter, J.W. Park, B.N. Ames, *Proc. Natl. Acad. Sci. U S A* 17, 6465 (1988).
18. M. Chee *et al.*, *Science* 274, 610 (1996).
19. S.A. Ahrendt *et al.*, *Proc. Natl. Acad. Sci. USA* 96, 7382 (1999).
20. Fragments containing mutations were PCR-amplified and then ethanol precipitated. For each mutation, discriminating oligonucleotides that contained the mutated base at the 3' end were designed (TAACCATA-3' (SEQ ID NO: 8) for patient #915 and TCTCTTACC-3' (SEQ ID NO: 9) for patient #898). Immediately adjacent [³²P] end-labeled 3' sequences (5'-CACACTACTA-3' (SEQ ID NO: 10) for patient #915 and 5'-TTAACCCAG-3' (SEQ ID NO: 11) for patient #898) were used as substrate together with discriminating oligonucleotides for the ligation reaction. After a denaturing step of 95°C for 5', the reactions were incubated for 1 hr at 37° in the presence of T4 DNA ligase (Life Technologies), in a buffer containing 50mM Tris-Cl, 10mM MgCl₂, 150mM NaCl, 1mM Spermidine, 1mM ATP,

5mM DTT, and analyzed on denatured 12% polyacrylamide gels. [Jen
et al., *Cancer Res.* 54, 5523 (1994)].

Table 1 Summary of mtDNA mutations

	Tumor*	Position	DNA	Protein	Gene
5	V478	710	T→C	-	12S rRNA
	"	1738	T→C	-	16S rRNA
	"	3308	T→C	M1T	ND1
	V429	8009	G→A	V142M	COX subunit II
	"	14985	G→A	R80H	CYT b
	"	15572	T→C	F276L	CYT b
10	V441	9949	G→A	V248I	COX subunit III
	V456	10563	T→C	C32R	ND4L
	V425	6264	G→A	G121trun	COX subunit I
	"	12418	insA	K28frameshif	ND5
	V451	1967	T→C	-	16S rRNA
	V410	2299	T→A	-	16S rRNA

15 *All the mutations were homoplasmic except V451 T11967C and V410 T2299A, which were present in ~50% of the mitochondrial DNA molecules.

Table 2. Summary of mitochondrial mutations in primary tumors.

Patient#	Location	Sequence	Protein	Gene
Bladder Cancer		(9/14, 57%)		
1124	114	T>C	N/C	D-loop
580	302	Del C	N/C	D-loop
580	386	C>A	N/C	D-loop
799	2056	G>A	N/C	16SrRNA
716	2445	T>C	N/C	16SrRNA
1127	3054	G>A	N/C	16SrRNA
884	10071	T>C	L-L	ND3
884	10321	T>C	V-A	ND3
884	10792	A>G	L-L	ND4
884	10793	C>T	L-L	ND4
899	10822	C>T	H-H	ND4
716	10978	A>G	L-L	ND4
870	11065	A>G	L-L	ND4
870	11518	G>A	L-L	ND4
884	12049	C>T	F-F	ND4
874	12519	T>C	V-V	ND5
580	15642	Del	7aa	Cyt b
899	16189	Ins T	N/A	D-loop
1124	16265	A>C	N/A	D-loop
1127	16532	A>T	N/A	D-loop
Lung Cancer		(6/15, 40%)		
1174	150	C>T	N/C	D-loop
1174	195	T>C	N/C	D-loop
902	302	Del C	N/C	D-loop
898	2664	T>C	N/C	16sRNA
915	5521	G>A	N/C	tRNATrp
915	12345	G>A	N/C	tRNALeu
915	16183	C>A	N/C	D-loop
915	16187	C>T	N/C	D-loop
1113	16519	T>C	N/C	D-loop
1140	16380	G>A	N/C	D-loop
Head and Neck Cancer		(6/13, 46%)		
1637	75	G>A	N/C	D-loop
1680	302	Ins C	N/C	D-loop
1565	514	Ins CG	N/C	D-loop
1708	10966	T>C	T>T	ND 4
1678	11150	G>A	A>T	ND 4
1680	16172	T>C	N/C	D-loop
1680	16292	C>T	N/C	D-loop
1680	16300	A>G	N/C	D-loop

Only D-loop region was analyzed for lung patients # 1113, #1140, and #1174

Table 3 New mtDNA polymorphisms (n = 57) found in this study.

Tumor	Position	Gene	Sequence change	
			DNA	Protein
B	633	tRNA Phe	A → G	-
B	723	12S rRNA	A → G	-
B, L, HNC	1738	16S rRNA	T → C	-
B	1872	16S rRNA	T → C	-
L	2308	16S rRNA	A → G	-
B, L	2395	16S rRNA	Del A	-
HNC	2712	16S rRNA	G → A	-
HNC	2758	16S rRNA	G → A	-
L	2768	16S rRNA	A → G	-
HNC	2768	16S rRNA	A → C	-
HNC	3148	16S rRNA	C → T	-
B, L, HNC	3308	ND1	T → C	M → T
B	4823	ND2	T → C	V → V
B	4917	ND2	A → G	N → D
B	5509	ND2	T → C	L → S
B	5567	tRNA Trp	T → C	-
B	5580	NCN	T → C	-
B	5899	NCN	Del C	-
B	6149	CoxI	A → G	L → L
B	6150	CoxI	G → A	V → I
B	6253	CoxI	T → C	M → T
B	6261	CoxI	G → A	A → T
B	6302	CoxI	A → G	A → A
B	7966	CoxII	C → T	F → F
B	8037	CoxII	G → A	R → H
B	8248	CoxII	A → G	M → M
B	8655	ATPase6	C → T	I → I
B	8877	ATPase6	T → C	F → F
B	9072	ATPase6	A → G	S → S
B	9093	ATPase6	A → G	T → T
B	9266	CoxIII	G → A	G → G
B	9497	CoxIII	T → C	F → F
L	10321	ND3	T → C	V → A
HNC	10403	ND3	A → G	E → E
B, L, HNC	10688	ND4L	G → A	V → V
B, L, HNC	10810	ND4	T → C	L → L
B	11164	ND4	A → G	R → R
L	11257	ND4	C → T	Y → Y
HNC	11339	ND4	T → C	L → L
L	11899	ND4	T → C	S → S
L	12519	ND5	T → C	V → V
B, L	14769	Cyt b	A → G	N → S
B	14992	Cyt b	T → C	L → L
L	15139	Cyt b	T → C	Y → Y
L	15514	Cyt b	T → C	Y → Y
L	15586	Cyt b	T → C	I → I
B	15601	Cyt b	T → C	P → P
L	15670	Cyt b	T → C	H → H
B	15672	Cyt b	T → C	M → T
B	15787	Cyt b	T → C	F → F
HNC	15941	tRNA Thr	T → C	-
L	15942	tRNA Thr	T → C	-
B	16130	D-Loop	G → A	-
L	16170	D-Loop	A → G	-
L	16204	D-Loop	G → C	-
L	16211	D-Loop	C → T	-
B	16225	D-Loop	C → T	-

B = Bladder cancer, L = Lung cancer, HNC = Head and neck cancer; NCN = non-coding nucleotide.

CLAIMS

1. A method of monitoring exposure of a person to an environmental pollutant, comprising the step of:
 - determining at two or more time points the presence of one or more mutations in mitochondrial DNA (mtDNA) in a body fluid of a person exposed to an environmental pollutant;
 - comparing the amount of mutations in mtDNA at different time points, wherein the amount of mutations correlates with amount of exposure to the environmental pollutant.
2. A method of monitoring exposure of a person to an environmental pollutant, comprising the step of:
 - measuring prevalence of one or more mutations in mitochondrial DNA (mtDNA) in a body fluid of a person exposed to an environmental pollutant, wherein a measured prevalence of one or more mutations in mtDNA of greater than 1% indicates clonal expansion of cells which harbor the one or more mutations in the person.
3. A method of monitoring exposure of a person to an environmental pollutant, comprising the step of:
 - measuring one or more mutations in a D-loop of mitochondrial DNA (mtDNA) in a body fluid of a person exposed to an environmental pollutant, wherein the number of mutations in mtDNA correlates with exposure to the environmental pollutant.
4. The method of claim 1, 2, or 3 wherein the body fluid is urine.
5. The method of claim 1, 2, or 3 wherein the body fluid is saliva.

6. The method of claim 1, 2, or 3 wherein the body fluid is sputum.
7. The method of claim 1, 2, or 3 wherein the body fluid is bronchoalveolar lavage.
8. The method of claim 1, 2, or 3 wherein mutations in mtDNA are measured with reference to mtDNA isolated from a normal tissue of the human.
9. The method of claim 8 wherein the normal tissue is paraffin-embedded.
10. The method of claim 1, 2, or 3 wherein mutations in mtDNA are measured with reference to mtDNA isolated from a blood, serum, or plasma sample of the human.
11. The method of claim 1 or 2 wherein mutations in mtDNA are measured by analysis of a gene encoding NADH dehydrogenase 4.
12. The method of claim 1 or 2 wherein mutations in mtDNA are measured by analysis of a gene encoding 16S rRNA.
13. The method of claim 2 wherein prevalence of one or more mutations in mtDNA in the body fluid is measured over time, whereby clonal expansion of cells can be monitored.
14. The method of claim 1 or 2 wherein mutations in mtDNA are measured by analysis of a cytochrome b gene.
15. The method of claim 1, 2, or 3 wherein the mutated mtDNA is found to bear a silent mutation.

16. The method of claim 1 or 2 wherein the mutated mtDNA is measured by analysis of non-coding regions.
17. The method of claim 1, 2, or 3 wherein the mutated mtDNA is measured by amplifying mtDNA segments of about 10 bp to about 4 kb.
18. The method of claim 1, 2, or 3 wherein the mutated mtDNA is measured by amplifying mtDNA segments of about 10 bp to about 2 kb.
19. The method of claim 1, 2, or 3 wherein the mutated mtDNA is measured by amplifying mtDNA segments of about 2 kb to about 4 kb.
20. The method of claim 1, 2, or 3 wherein the mutated mtDNA is measured by an oligonucleotide mismatch ligation assay.
21. The method of claim 1, 2, or 3 wherein the environmental pollutant is cigarette smoke.
22. The method of claim 1, 2, or 3 wherein the environmental pollutant is a biological toxin.
23. The method of claim 1, 2, or 3 wherein the environmental pollutant is radiation.
24. The method of claim 1, 2, or 3 wherein the environmental pollutant is industrial waste.
25. The method of claim 1, 2, or 3 wherein the environmental pollutant is chemical.
26. The method of claim 1, 2, or 3 wherein the environmental pollutant is water-borne.

27. The method of claim 1, 2, or 3 wherein the environmental pollutant is air-borne.
28. The method of claim 1, 2, or 3 wherein the environmental pollutant is a drug.
29. A kit for monitoring exposure of a person to environmental pollutants, comprising:
 - one or more primers which hybridize to a mitochondrial D-loop for making a primer extension product; and
 - written material identifying mutations which are found in the D-loop as a result of exposure to one or more environmental pollutants.
30. The kit of claim 29 further comprising a buffer.
31. The kit of claim 29 further comprising nucleic acid probes for hybridization to the extension product.
32. The kit of claim 29 wherein the primers are for PCR.
33. The kit of claim 29 wherein the primers are for a ligation assay.
34. The method of claim 3 wherein the mutation is selected from the group consisting of: T_{OC} at nucleotide 114; ΔC at nucleotide 302; C_{OA} at nucleotide 386; insert T at nucleotide 16189; A_{OC} at nucleotide 16265; A_{OT} at nucleotide 16532; C_{OT} at nucleotide 150; T_{OC} at nucleotide 195; ΔC at nucleotide 302; C_{OA} at nucleotide 16183; C_{OT} at nucleotide 16187; T_{OC} at nucleotide 16519; G_{OA} at nucleotide 16380; G_{OA} at nucleotide 75; insert C at nucleotide 302; insert CG at nucleotide 514; T_{OC} at nucleotide 16172; C_{OT} at nucleotide 16292; and A_{OG} at nucleotide 16300.

35. The kit of claim 29 wherein the written material identifies a mutation selected from the group consisting of: T_{OC} at nucleotide 114; ΔC at nucleotide 302; C_{OA} at nucleotide 386; insert T at nucleotide 16189; A_{OC} at nucleotide 16265; A_{OT} at nucleotide 16532; C_{OT} at nucleotide 150; T_{OC} at nucleotide 195; ΔC at nucleotide 302; C_{OA} at nucleotide 16183; C_{OT} at nucleotide 16187; T_{OC} at nucleotide 16519; G_{OA} at nucleotide 16380; G_{OA} at nucleotide 75; insert C at nucleotide 302; insert CG at nucleotide 514; T_{OC} at nucleotide 16172; C_{OT} at nucleotide 16292; and A_{OG} at nucleotide 16300.
36. The method of claim 11 wherein the mutation is selected from the group consisting of: A G at nucleotide 10792, C T at nucleotide 10793, C T at nucleotide 10822, A G at nucleotide 10978, A G at nucleotide 11065, G A at nucleotide 11518, C T at nucleotide 12049, T C at nucleotide 10966, and G A at nucleotide 11150.
37. The method of claim 12 wherein the mutation is selected from the group consisting of: G A at nucleotide 2056, T C at nucleotide 2445, T C at nucleotide 2664, and G A at nucleotide 3054.
38. The method of claim 14 wherein the mutation is **Δ 7 amino acids at nucleotide 15642.**
39. An oligonucleotide probe comprising a sequence of at least 10 contiguous nucleotides of a human mitochondrial genome, wherein the oligonucleotide comprises a mutation selected from the group consisting of: a mutation selected from the group consisting of: T_{OC} at nucleotide 114; ΔC at nucleotide 302; C_{OA} at nucleotide 386; insert T at nucleotide 16189; A_{OC} at nucleotide 16265; A_{OT} at nucleotide 16532; C_{OT} at nucleotide 150; T_{OC} at nucleotide 195; ΔC at nucleotide 302; C_{OA} at nucleotide 16183; C_{OT} at nucleotide 16187; T_{OC} at nucleotide 16519; G_{OA} at nucleotide 16380; G_{OA} at nucleotide 75; insert C at nucleotide 302; insert CG at nucleotide 514; T_{OC} at nucleotide 16172; C_{OT} at nucleotide 16292; A_{OG} at

nucleotide 16300; A G at nucleotide 10792; C T at nucleotide 10793; C T at nucleotide 10822; A G at nucleotide 10978; A G at nucleotide 11065; G A at nucleotide 11518; C T at nucleotide 12049; T C at nucleotide 10966; G A at nucleotide 11150; G A at nucleotide 2056; T C at nucleotide 2445; T C at nucleotide 2664; T C at nucleotide 10071; T C at nucleotide 10321; T C at nucleotide 12519; Δ 7 amino acids at nucleotide 15642; G -A at nucleotide 5521; G A at nucleotide 12345; G A at nucleotide 3054; TÝC substitution at position 710; TÝC substitution at position 1738; TÝC substitution at position 3308; GÝA substitution at position 8009; GÝA substitution at position 14985; TÝC substitution at position 15572; GÝA substitution at position 9949; TÝC substitution at position 10563; GÝA substitution at position 6264; A insertion at position 12418; TÝC substitution at position 1967; and TÝA substitution at position 2299.

40. An oligonucleotide primer comprising a sequence of at least 10 contiguous nucleotides of a human mitochondrial genome, wherein the oligonucleotide comprises a mutation selected from the group consisting of: a mutation selected from the group consisting of: TOC at nucleotide 114; ΔC at nucleotide 302; CÖA at nucleotide 386; insert T at nucleotide 16189; AÖC at nucleotide 16265; AÖT at nucleotide 16532; CÖT at nucleotide 150; TÖC at nucleotide 195; ΔC at nucleotide 302; CÖA at nucleotide 16183; CÖT at nucleotide 16187; TÖC at nucleotide 16519; GÖA at nucleotide 16380; GÖA at nucleotide 75; insert C at nucleotide 302; insert CG at nucleotide 514; TÖC at nucleotide 16172; CÖT at nucleotide 16292; AÖG at nucleotide 16300; A G at nucleotide 10792; C T at nucleotide 10793; C T at nucleotide 10822; A G at nucleotide 10978; A G at nucleotide 11065; G A at nucleotide 11518; C T at nucleotide 12049; T C at nucleotide 10966; G A at nucleotide 11150; G A at nucleotide 2056; T C at nucleotide 2445; T C at nucleotide 2664; T C at nucleotide 10071; T C at nucleotide 10321; T C at nucleotide 12519; Δ 7 amino acids at nucleotide 15642; G -A at

nucleotide 5521; G A at nucleotide 12345; G A at nucleotide 3054; TÝC substitution at position 710; TÝC substitution at position 1738; TÝC substitution at position 3308; GÝA substitution at position 8009; GÝA substitution at position 14985; TÝC substitution at position 15572; GÝA substitution at position 9949; TÝC substitution at position 10563; GÝA substitution at position 6264; A insertion at position 12418; TÝC substitution at position 1967; and TÝA substitution at position 2299.

41. A method to aid in detecting the presence of tumor cells in a patient, comprising:

determining the presence of a single basepair mutation in a mitochondrial genome of a cell sample of a patient, wherein the mutation is found in a tumor of the patient but not in normal tissue of the patient, wherein the tumor is not a colorectal tumor; and

identifying the patient as having a tumor if one or more single basepair mutations are determined in the mitochondrial genome of the cell sample of the patient.

42. A method to aid in detecting the presence of tumor cells in a patient, comprising:

determining the presence of a mutation in a D-loop of a mitochondrial genome of a cell sample of a patient, wherein the mutation is found in a tumor of the patient but not in normal tissue of the patient; and

identifying the patient as having a tumor if one or more single basepair mutations are determined in the mitochondrial genome of the cell sample of the patient.

43. The method of claim 42 wherein the mutation is selected from the group consisting of: TÖC at nucleotide 114; ΔC at nucleotide 302; CÖA at nucleotide 386; insert T at nucleotide 16189; AÖC at nucleotide 16265; AÖT at nucleotide 16532; CÖT at nucleotide 150; TÖC at

nucleotide 195; ΔC at nucleotide 302; C̄ŌA at nucleotide 16183; C̄ŌT at nucleotide 16187; T̄ŌC at nucleotide 16519; ḠŌA at nucleotide 16380; ḠŌA at nucleotide 75; insert C at nucleotide 302; insert CG at nucleotide 514; T̄ŌC at nucleotide 16172; C̄ŌT at nucleotide 16292; and ĀŌG at nucleotide 16300.

44. A method to aid in detecting the presence of tumor cells in a patient, comprising:

determining the presence of a single basepair mutation in a mitochondrial genome of a cell sample of a patient, wherein the mutation is found in a cancer of the patient but not in normal tissue of the patient, wherein the cancer is selected from the group of cancers consisting of: lung, head and neck, bladder, brain, breast, lymphoma, leukaemia, skin, prostate, stomach, pancreas, liver, ovarian, uterine, testicular, and bone; and

identifying the patient as having a tumor if one or more single basepair mutations are determined in the mitochondrial genome of the cell sample of the patient.

45. A method to aid in detecting the presence of tumor cells in a patient, comprising:

step for determining the presence of a single basepair mutation in a mitochondrial genome of a cell sample of a patient, wherein the mutation is found in a tumor of the patient but not in normal tissue of the patient, wherein the cancer is selected from the group of cancers consisting of: lung, head and neck, and bladder; and

identifying the patient as having a tumor if one or more single basepair mutations are determined in the mitochondrial genome of the cell sample of the patient.

46. A method to aid in detecting the presence of tumor cells in a patient, comprising:

determining the presence of a mutation in a mitochondrial genome of a cell sample of a patient, wherein the **mutation is selected from the group consisting of:** T̄ŌC at nucleotide 114; ΔC at

nucleotide 302; C₀A at nucleotide 386; insert T at nucleotide 16189; A₀C at nucleotide 16265; A₀T at nucleotide 16532; C₀T at nucleotide 150; T₀C at nucleotide 195; ΔC at nucleotide 302; C₀A at nucleotide 16183; C₀T at nucleotide 16187; T₀C at nucleotide 16519; G₀A at nucleotide 16380; G₀A at nucleotide 75; insert C at nucleotide 302; insert CG at nucleotide 514; T₀C at nucleotide 16172; C₀T at nucleotide 16292; A₀G at nucleotide 16300; A G at nucleotide 10792; C T at nucleotide 10793; C T at nucleotide 10822; A G at nucleotide 10978; A G at nucleotide 11065; G A at nucleotide 11518; C T at nucleotide 12049; T C at nucleotide 10966; G A at nucleotide 11150; G A at nucleotide 2056; T C at nucleotide 2445; T C at nucleotide 2664; T C at nucleotide 10071; T C at nucleotide 10321; T C at nucleotide 12519; Δ 7 amino acids at nucleotide 15642; G A at nucleotide 5521; G A at nucleotide 12345; and G A at nucleotide 3054; and

identifying the patient as having a tumor if one or more mutations are determined in the mitochondrial genome of the cell sample of the patient.

47. The method of claim 41, 42, 44, 45, or 46 wherein the cell sample is from blood.

48. The method of claim 41, 42, 44, 45, or 46 wherein the cell sample is from urine.

49. The method of claim 41, 42, 44, 45, or 46 wherein the cell sample is from sputum.

50. The method of claim 41, 42, 44, 45, or 46 wherein the cell sample is from saliva.

51. The method of claim 41, 42, 44, 45, or 46 wherein the cell sample is from feces.
52. The method of claim 41, 42, 44, 45, or 46 wherein the step for determining comprises amplifying mitochondrial DNA.
53. The method of claim 41, 42, 44, 45, or 46 wherein the step for determining comprises sequencing mitochondrial DNA.
54. The method of claim 41, 42, 44, 45, or 46 wherein the step for determining comprises hybridization of DNA amplified from the mitochondrial genome of the cell sample to an array of oligonucleotides which comprises matched and mismatched sequences to human mitochondrial genomic DNA.
55. The method of claim 41, 42, 44, 45, or 46 wherein the mutation is a substitution mutation.
56. The method of claim 41, 42, 44, 45, or 46 wherein the mutation is a one basepair insertion.
57. The method of claim 41, 42, 44, 45, or 46 wherein the mutation is a one basepair deletion.
58. The method of claim 41, 42, 44, 45, or 46 wherein the mutation is a transition mutation.
59. The method of claim 41, 42, 44, 45, or 46 wherein the mutation is a homoplasmic mutation.
60. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a T>C substitution at position 710.

61. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a TÝC substitution at position 1738.
62. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a TÝC substitution at position 3308.
63. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a GÝA substitution at position 8009.
64. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a GÝA substitution at position 14985.
65. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a TÝC substitution at position 15572.
66. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a GÝA substitution at position 9949.
67. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a TÝC substitution at position 10563.
68. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a GÝA substitution at position 6264.
69. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is an A insertion at position 12418.
70. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a TÝC substitution at position 1967.

71. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a TÝA substitution at position 2299.
72. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a T C at nucleotide 10071.
73. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a T C at nucleotide 10321.
74. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a T C at nucleotide 12519.
75. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a G A at nucleotide 5521.
76. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a G A at nucleotide 12345.
77. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a TÔC at nucleotide 114.
78. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a Δ C at nucleotide 302.
79. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a CÔA at nucleotide 386.
80. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is an insert T at nucleotide 16189.
81. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a AÔC at nucleotide 16265.

82. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **ĀT at nucleotide 16532**.
83. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **C̄T at nucleotide 150**.
84. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **T̄C at nucleotide 195**.
85. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **ΔC at nucleotide 302**.
86. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **C̄A at nucleotide 16183**.
87. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **C̄T at nucleotide 16187**.
88. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **T̄C at nucleotide 16519**.
89. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **ḠA at nucleotide 16380**.
90. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **ḠA at nucleotide 75**.
91. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is an **insert C at nucleotide 302**.
92. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is an **insert CG at nucleotide 514**.

93. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **T**~~O~~**C** at **nucleotide 16172**.
94. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **C**~~O~~**T** at **nucleotide 16292**.
95. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is an **A**~~O~~**G** at **nucleotide 16300**.
96. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is an **A** **G** at **nucleotide 10792**.
97. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **C** **T** at **nucleotide 10793**.
98. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **C** **T** at **nucleotide 10822**.
99. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **A** **G** at **nucleotide 10978**.
100. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **A** **G** at **nucleotide 11065**.
101. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **G** **A** at **nucleotide 11518**.
102. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **C** **T** at **nucleotide 12049**.

103. The oligonucleotide probe of claim 39 or primer of claim 40
wherein the mutation is a **T C** at nucleotide 10966.

104. The oligonucleotide probe of claim 39 or primer of claim 40
wherein the mutation is a **G A** at nucleotide 11150.

105. The oligonucleotide probe of claim 39 or primer of claim 40
wherein the mutation is a **G A** at nucleotide 2056.

106. The oligonucleotide probe of claim 39 or primer of claim 40
wherein the mutation is a **T C** at nucleotide 2445.

107. The oligonucleotide probe of claim 39 or primer of claim 40
wherein the mutation is a **T C** at nucleotide 2664.

108. The oligonucleotide probe of claim 39 or primer of claim 40
wherein the mutation is a **T C** at nucleotide 10071.

109. The oligonucleotide probe of claim 39 or primer of claim 40
wherein the mutation is a **T C** at nucleotide 10321.

110. The oligonucleotide probe of claim 39 or primer of claim 40
wherein the mutation is a **T C** at nucleotide 12519.

111. The oligonucleotide probe of claim 39 or primer of claim 40
wherein the mutation is a **Δ 7 amino acids** at nucleotide 15642.

112. The oligonucleotide probe of claim 39 or primer of claim 40
wherein the mutation is a **G A** at nucleotide 5521.

113. The oligonucleotide probe of claim 39 or primer of claim 40
wherein the mutation is a **G A** at nucleotide 12345.

114. The oligonucleotide probe of claim 39 or primer of claim 40 wherein the mutation is a **G A** at nucleotide 3054.

115. The method of claim 2 wherein the mutation was identified previously in a tumor of the patient.

116. The method of claim 115 wherein the patient has received anti-cancer therapy and the step for determining is performed at least three times to monitor progress of the anti-cancer therapy.

117. The method of claim 1 further comprising a step for testing a normal tissue of the patient to determine the absence of the mutation.

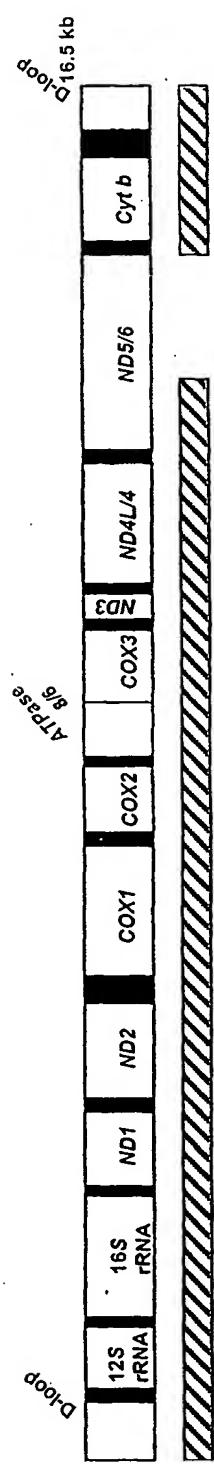


Fig. 1.

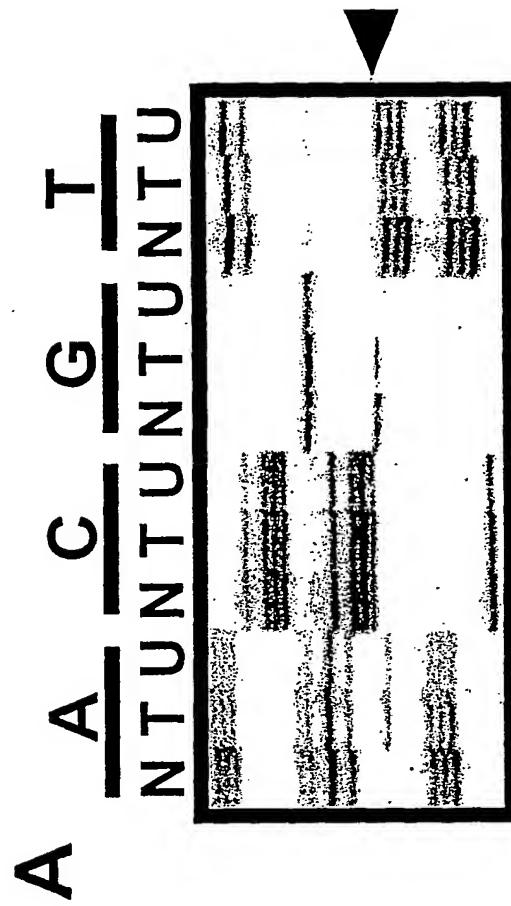


Fig. 2A.

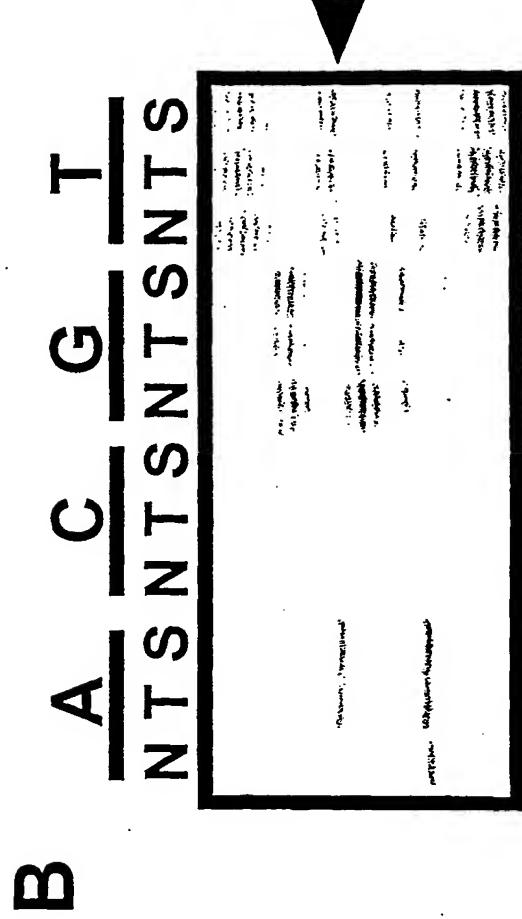


Fig. 2B.

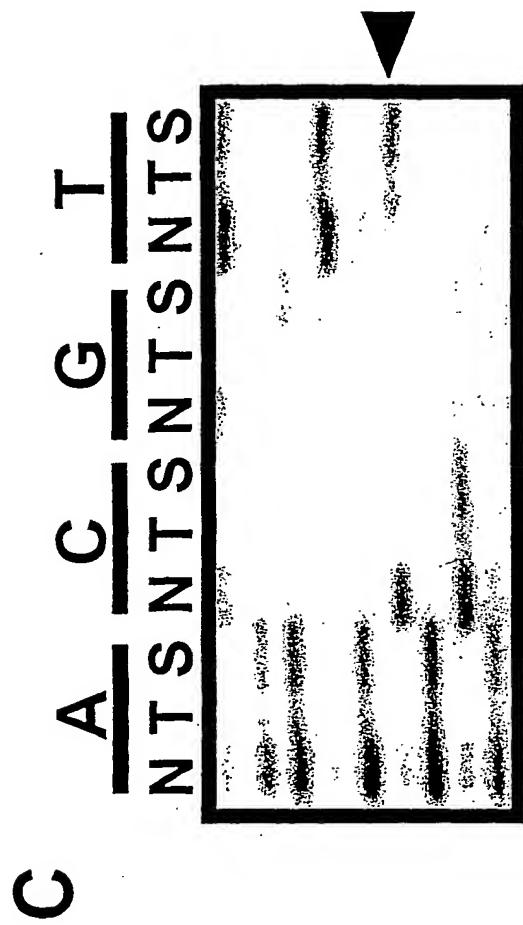


Fig. 2C.

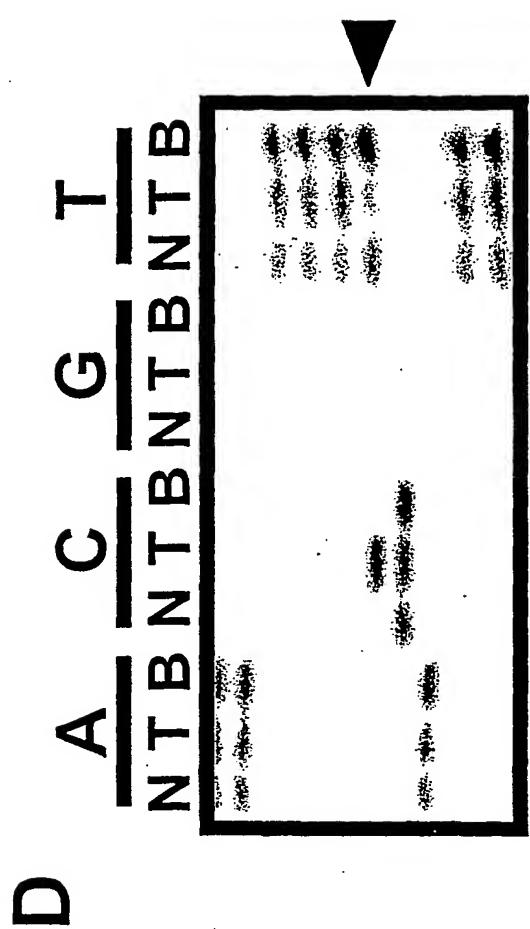


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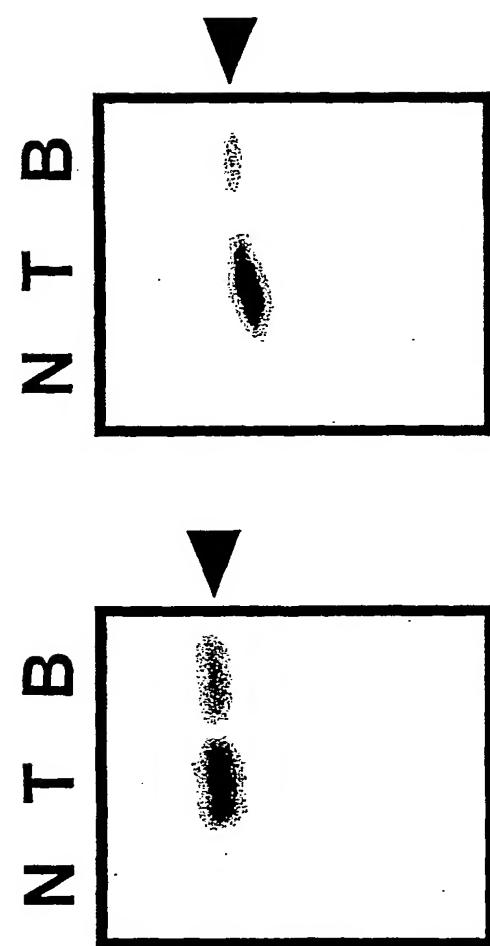


Fig. 3.

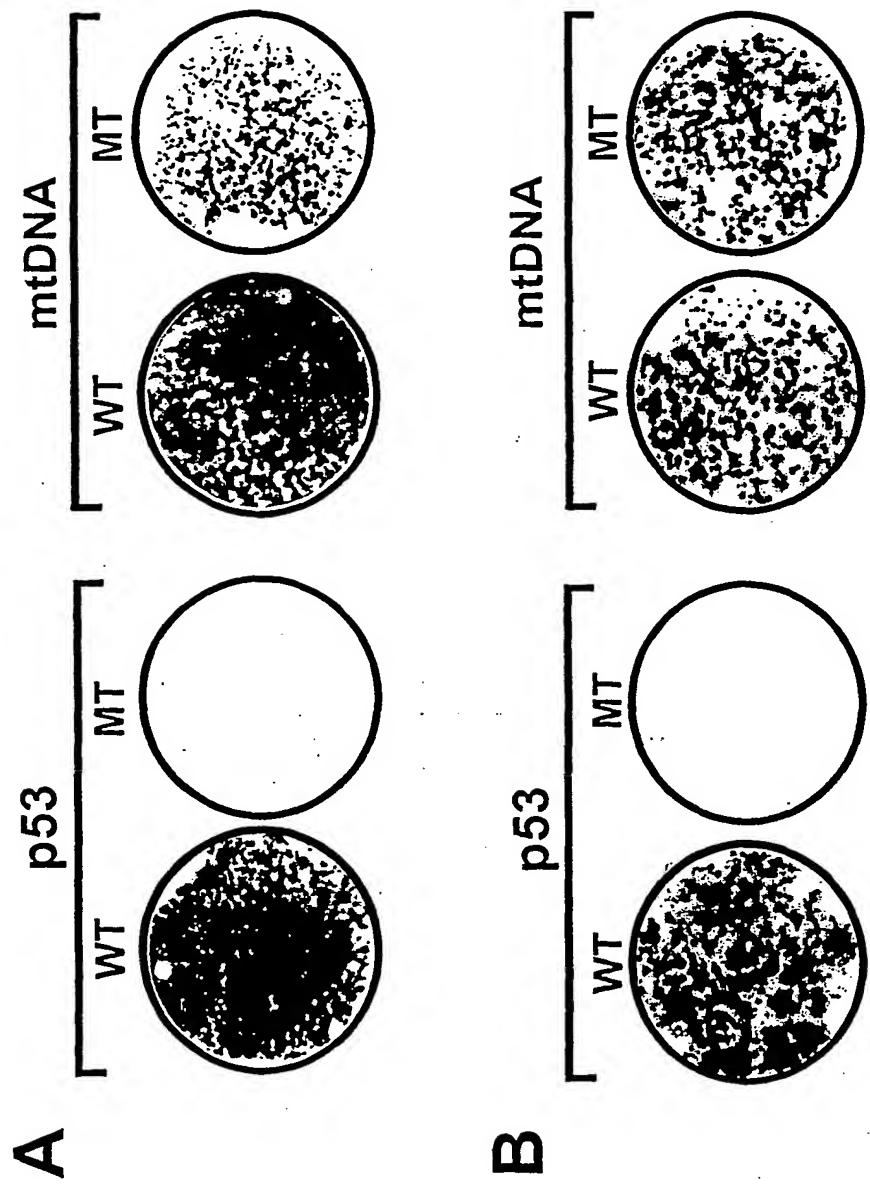


Fig. 4.

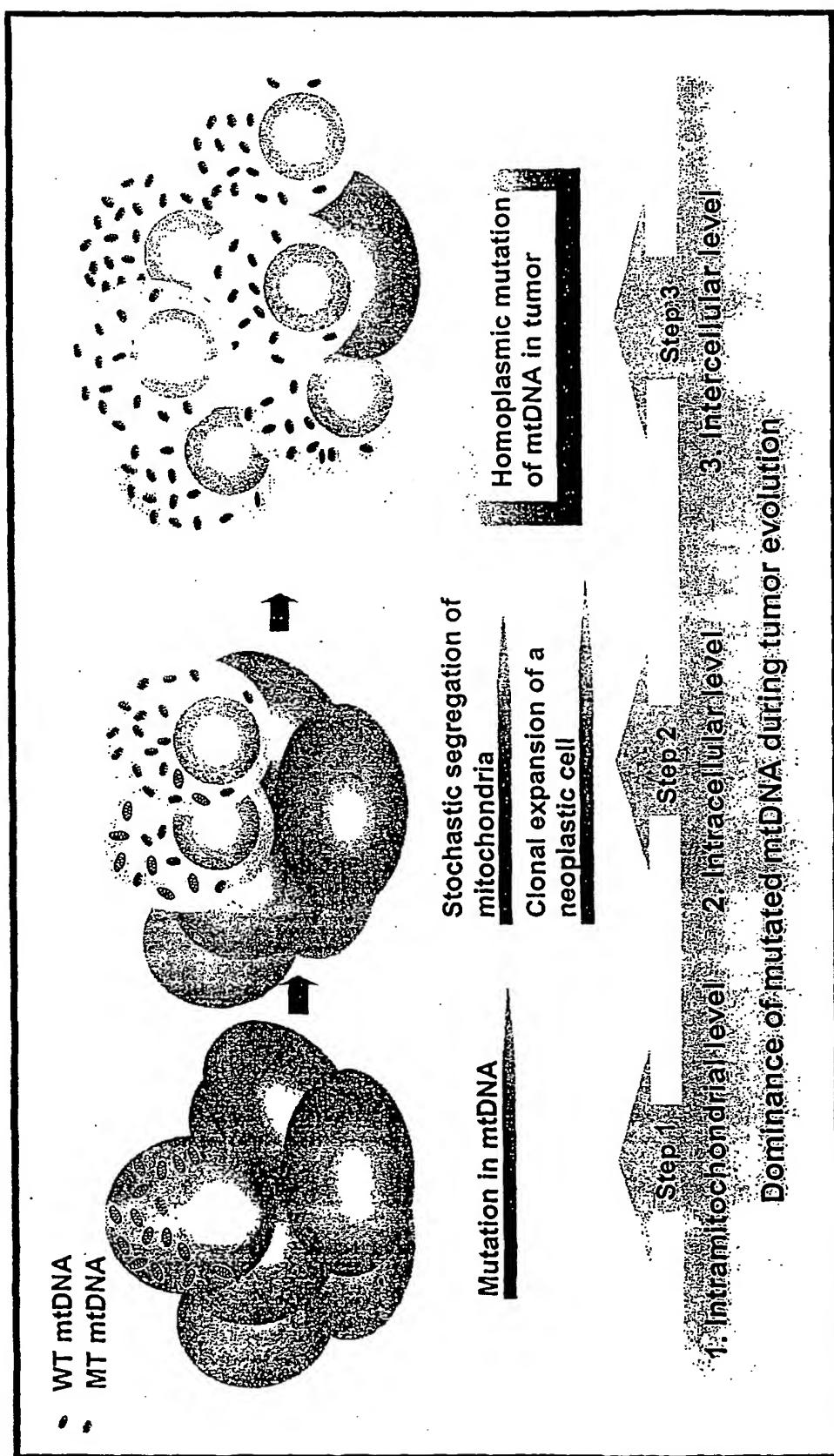


Figure 5

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(21) International Application Number: PCT/US01/08253

(74) Agents: KAGAN, Sarah, A. et al.; Banner & Witcoff, Ltd., Eleventh floor, 1001 G Street, N.W., Washington, DC 20001-4597 (US).

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(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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Published:
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(88) Date of publication of the international search report:
10 April 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 01/068923 A3

(54) Title: MITOCHONDRIAL DOSIMETER

(57) Abstract: Mitochondrial mutations occur as a product of contact of a person with an environmental pollutant. Mitochondrial mutations are readily detectable in body fluids. Measurement of mitochondrial mutations in body fluids can be used as a dosimeter to monitor exposure to the environmental pollutant. Mitochondrial mutations can also be detected in cancer patients. Probes and primers containing mutant mitochondrial sequences can be used to monitor patient condition.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/08253

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, SEQUENCE SEARCH, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BURGART L J ET AL: "SOMATIC MITOCHONDRIAL MUTATION IN GASTRIC CANCER", AMERICAN JOURNAL OF PATHOLOGY, PHILADELPHIA, PA, US, vol. 147, October 1995 (1995-10), pages 1105-1111, XP000863263 ISSN: 0002-9440 * whole document *	1-117
X	BIANCHI M S ET AL: "Mitochondrial DNA mutations in normal and tumor tissues from breast cancer patients.", CYTOGENETICS AND CELL GENETICS, vol. 71, no. 1, 1995, pages 99-103, XP008006976 ISSN: 0301-0171 * whole document *	1-117

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority, claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

27 August 2002

09.12.2002

Name and mailing address of the ISA

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Authorized officer

Aguilera, M

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/08253

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALONSO ANTONIO ET AL: "Detection of somatic mutations in the mitochondrial DNA control region of colorectal and gastric tumors by heteroduplex and single-strand conformation analysis." ELECTROPHORESIS, vol. 18, no. 5, 1997, pages 682-685, XP002210338 ISSN: 0173-0835 * whole document * ---	1-117
X	TAMURA G ET AL: "Mutations in mitochondrial control region DNA in gastric tumours of Japanese patients." EUROPEAN JOURNAL OF CANCER, vol. 35, no. 2, February 1999 (1999-02), pages 316-319, XP001100623 ISSN: 0959-8049 * whole document * ---	1-117
X	WO 00 11219 A (KINZLER KENNETH W ;POLYAK KORNELIA (US); VOGELSTEIN BERT (US); UNI) 2 March 2000 (2000-03-02) page 6, line 23 -page 7, line 14 page 7, line 32 -page 9, line 15; example 2 page 5, line 25 -page 6, line 10; example 1 ---	1-117
X	LIU CHIN-SAN ET AL: "Smoking-associated mitochondrial DNA mutations in human hair follicles." ENVIRONMENTAL AND MOLECULAR MUTAGENESIS, vol. 30, no. 1, 1997, pages 47-55, XP000806982 ISSN: 0893-6692 * Abstract, Materials and Methods * ---	1-117
X	BALLINGER S W ET AL: "Mitochondrial genome damage associated with cigarette smoking." CANCER RESEARCH. UNITED STATES 15 DEC 1996, vol. 56, no. 24, 15 December 1996 (1996-12-15), pages 5692-5697, XP001074281 ISSN: 0008-5472 * Abstract, Introduction * ---	1-117
X	DATABASE EMBL H. sapiens CpG island DNA genomic (185 nt), 17 October 1995 (1995-10-17) Database accession no. Z56152 XP002211247 the whole document ---	1-117

-/-

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/08253

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WALLACE D C: "MITOCHONDRIAL DISEASES IN MAN AND MOUSE" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE, US, vol. 283, 5 March 1999 (1999-03-05), pages 1482-1488, XP002937354 ISSN: 0036-8075 -----	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 01/08253

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: 1-117 (all partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-117 (all partially)

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-117 (all partially)

The large number of the claims presently on file, the presence of several independent claims with 38 options, and also the complex dependency structure, render it difficult to determine the matter for which protection is sought. The ISA is of the opinion that the present application fails to comply with the clarity and conciseness requirements of PCT Article 6 (see also PCT Rule 6.1(a)) to such an extent that a meaningful search on the basis of the claims is impossible, especially with regard to the establishment of the claims relating to each one of the multiple inventions found in the application (see enclosed Invitation to pay additional fees).

Consequently, the search has been carried out for what appear to be the clear and concise concepts of the application, namely oligonucleotide primers and probes comprising the mitochondrial mutations listed, kits comprising such probes, and methods using them.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-117 (all partially)

INVENTION 1:

Oligonucleotide primers and probes comprising the mitochondrial mutation T114C, kits comprising such primers, and methods using them.

2. Claims: 1-117 (all partially)

INVENTIONS 2-38:

Oligonucleotide primers and probes comprising each one of the mitochondrial mutations listed in Table 2 (page 23), kits comprising such primers, and methods using them. Invention 2 relates to mutation Del302C, invention 3 relates to mutation C386A, etc.

3. Claims: 1-117 (all partially)

INVENTIONS 39-50:

Oligonucleotide primers and probes comprising each one of the mitochondrial mutations listed in Table 1 (page 22), kits comprising such primers, and methods using them. Invention 39 relates to mutation T710C, invention 40 relates to mutation T1738C, etc.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 01/08253

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 0011219	A	02-03-2000	AU 5677899 A	14-03-2000
		CA 2340175 A1		02-03-2000
		EP 1104492 A1		06-06-2001
		JP 2002523061 T		30-07-2002
		NO 20010843 A		18-04-2001
		US 2002164622 A1		07-11-2002
		WO 0011219 A1		02-03-2000
		US 6344322 B1		05-02-2002

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